METHOD FOR MODIFYING PLANTS

Field of invention

This invention relates to a method for the modification of plants, more specifically to a process for increasing the level of certain isoprenoid compounds, specifically sterols, in plants.

Background of the invention

Many approaches have been suggested for modifying isoprenoid production in plants.

Whereas only a few sterols exist in animals, with cholesterol being by far the major one, in plants a wide range of sterols is found. Structural variations between these arise from different substitutions in the side chain and the number and position of double bonds in the tetracyclic skeleton.

Plant sterols can be grouped by the presence or absence of one or more functionalities. For example they can be divided into three groups based on methylation levels at C4 as follows: 4-desmethylsterols or end product sterols, 4-monomethylsterols and 4,4-dimethylsterols. Naturally occurring 4-desmethylsterols include sitosterol, stigmasterol, brassicasterol, Δ7-avenosterol and campesterol. The 4,4-dimethyl sterols include cycloartenol and 24-methylene cycloartanol and the 4-monomethyl sterols

include 24-methylene lophenol and 24-ethylidene lophenol. In most higher plants, sterols with a free 3-hydroxyl group (free sterols) are the major end products. However, sterols also occur as conjugates, for example, where the 3-hydroxy group is esterified by a fatty acid chain, phenolic acids or sugar moieties to give sterol esters. For the purpose of this description, the term sterol refers both to free sterols and conjugated sterols. However, in this specification references to levels, amounts or percentages of sterol refer to the total weight of sterol groups whereby the weight of the conjugating groups such as fatty acid, phenolic acid or sugar groups is excluded.

To date, most studies aimed at manipulating sterols in plants have involved other than 4-desmethylsterols with the purpose of increasing resistance to pests or to fungicides.

WO 98/45457 describes the modulation of phytosterol compositions to confer resistance to insects, nematodes, fungi and/or environmental stresses, and/or to improve the nutritional value of plants by using a double stranded DNA molecule comprising a promoter, a DNA sequence encoding a first enzyme which binds a first sterol and produces a second sterol and a 3' non-translated region which causes polyadenylation at the 3' end of the RNA. Preferably the enzyme is selected from the group consisting of S-adenosyl-L-methionine-$\Delta^{24(25)}$-sterol methyl transferase, a C-4 demethylase, a cycloeucalenol to obtusifoliol-isomerase, a 14-α-demethylase, a Δ^{8} to Δ^{7}- isomerase, a Δ^{7}-C-5-desaturase and a 24,25-reductase. The only mention of a C-4 demethylase is in the context of inhibiting this enzyme in order to confer resistance to insects. There is no use described of a C-4 demethylase in the context of nutrition.

US 5,306,862 describes a method of increasing sterol accumulation in a plant by increasing the copy number of a gene encoding a polypeptide having HMG-CoA reductase activity to increase the resistance of plants to pests. Similarly US 5,349,126 discloses a process to increase the squalene and sterol accumulation in transgenic plants by increasing the amount of a gene encoding a polypeptide having HMG-CoA reductase activity to increase the pest resistance of transgenic plants.

Gondet et al in Plant Physiology (1994) 105:509-518 has isolated a tobacco mutant showing dramatically altered sterol compositions in leaf tissue with significant increases in the proportion of cyclopropylsterols and HMGR activities increased by approximately 3-fold.

Re et al in The Plant Journal (1995) 7(5), 771-784 have shown that the over-expression of *Arabidopsis thaliana* HMG CoA reductase (HMG 1) is not sufficient to alter the bulk synthesis and accumulation of end products of the plant isoprenoid pathway.

In plants, 24-methylene cycloartanol production from cycloartenol via sterol methyltransferase1 (SMT1) is one of the steps in isoprenoid biosynthesis.

Bouvier-Nave et al in Eur. J. Biochem. 256, 88-96 (1988) describes two families of sterol methyl transferases (SMTs), The first (SMT1) applying to cycloartenol and the second (SMT2) to 24-methylene lophenol.

Schaller et al in Plant Physiology (1998) 118: 461-169 describes the over-expression of SMT2 from Arabidopsis in tobacco resulting in a change in the ratio of 24-methyl cholesterol to sitosterol in the tobacco leaf.

Diener et al in The Plant Cell (2000) 12: 853-870 describes the functional characterisation of an Arabidopsis SMT1 gene and show that mutants lacking the gene display poor growth and fertility.

Schaeffer et al in Lipids (2000) 35: 263-269 describe the effects of expressing *Nicotiana tabacum* SMT1 and SMT2 genes in transgenic tobacco. Overexpression of SMT1 results in variations in the level of cycloartenol and concomitant changes in the proportion of 24-ethyl sterols. Over expression of SMT 2 alters the ratio of 24-methyl cholesterol to sitosterol resulting in reduced growth.

WO 01/31027 discloses the use of non-feedback regulated HMGR genes in sterol production.

WO 01/79513 describes the use of SMT1 genes in sterol production.

A sterol over-producing tobacco mutant has been shown to over-accumulate cycloartenol (CA), 24-methylenecycloartanol (24MCA) and 24-ethylidene lophenol (24Eloph) (Maillot-Vernier et al. Mol. Biol. Genet. 231: 33-40, 1991).

The conversion of 24MCA to cycloeucalenol is the first of three C4 demethylation reactions that take place during sterol biosynthesis (Figure 1). This involves the removal of the α-methyl group at the C4 position of the sterol

backbone. The other demethylation steps, the removal of the C4β methyl group, are the conversions of 24-methylene lophenol (24Mloph) to episterol and 24Eloph to Δ7-avenasterol (Figure 1).

In animal and yeast systems the removal of the sterol C4 methyl groups are well characterised (Faust et al., Biology of Cholesterol, ed. Yeagle, P.L. (CRC, Boca Raton, FL, USA) p. 19-38, 1988; Bard et al., Proc. Natl. Acad. Sci. USA 93: 186-190, 1996). The first step in the C4 demethylation of 4,4-dimethylzymosterol (4,4-DMZ) in yeast is catalysed by a C4 sterol methyl oxidase (ERG25) and involves the oxidation of the C4α methyl group to give a carboxylic acid. In the subsequent step the carboxyl group is removed by a C4 decarboxylase (ERG26) resulting in a keto group at the C3 position (Gachotte et al., Proc. Natl. Acad. Sci. USA, 96: 12655-12660, 1999). Finally, a C3-keto reductase (ERG27) reduces the keto group to give an alcohol group at the C3 position (Gachotte et al., Proc. Natl. Acad. Sci. USA, 96: 1810 1999). These catalytic steps are repeated to remove the C4β methyl group on the sterol backbone. In contrast to yeast, the mechanism for removing the two methyl groups at the C4 position has yet to be fully elucidated in plants. It has however been shown that plants have at least two distinct microsomal C4 demethylation complexes involved in sterol biosynthesis (Pascal et al, J. Biol. Chem. 268: 11639-11654, 1993).

WO 02/42477 discloses that expressing genes encoding specific HMG-reductase enzymes (HMGR) in combination with those encoding sterol methyltransferase1 can advantageously be used to further increase the nutritional value of plants especially in the seeds thereof. Specifically, the use of

non-feedback regulated HMGR in combination with overexpression of sterol methyltransferase1 leads to the further enhancement of nutritionally beneficial sterol for example in the seeds of said plants compared to plants where only one of the above genes has been expressed.

It has been found that over-expression of HMGR and HMGR/SMT1, according to the technique described above, results in the accumulation of not only end-product sterols but also certain sterol intermediates. Several of these intermediates are of the C4 di-methyl or mono-methyl type (e.g. 24-methylene cycloartanol, 24-methylene lophenol and 24-ethylidene lophenol). It would therefore be valuable if such sterol intermediates could be converted to end product sterols (4-desmethyl sterols). C4 di- or mono-methyl sterol is demethylated in a three-step reaction catalysed by three separate enzymes (C4-sterol methyl oxidase, C4-sterol decarboxylase and C3-sterol ketoreductase).

The present invention aims to modify sterol levels in plants, especially the seeds of plants whereby this modification can either involve an increase of the level of (beneficial) sterols or a decrease of the level of (less-desired) sterols such as cholesterol.

The present invention also aims to increase sterol levels in plants, whereby the sterols are preferably nutritionally attractive 4-desmethylsterols such as sitosterols, stigmasterols, brassicasterol, Δ7-avenosterol or campesterols and whereby the sterols are preferably expressed in the seeds.

It is a further aim of the present invention to increase the level of 4-desmethylsterols in plants that are modified compared to the corresponding wild type plant by over-expression of HMGR and/or SMT1.

Statement of the invention

According to the present invention, there is provided a method for increasing the level of 4-desmethyl sterols in a plant which comprises increasing the enzymatic demethylation of 4-monomethyl and 4,4-dimethyl sterols.

In another aspect, the invention relates to a plant having increased levels of 4-desmethyl sterols compared to the wild type plant in which the levels are increased according to the method of the invention. Also provided by the invention in another aspect is plant material obtainable from the plant of the invention.

A further aspect of the invention is a method of transforming a plant which comprises:

(a) transforming a plant cell with a recombinant DNA construct comprising a DNA segment encoding a polypeptide with C4SMO activity and a promoter for driving expression of said polypeptide in said plant cell, to form a transformed plant cell;

(b) regenerating the transformed plant cell into a transgenic plant; and

(c) selecting transgenic plants that have enhanced levels of 4-desmethyl sterols compared to wild type strains of the same plant.

Another aspect of the invention is a process for producing an oil comprising a 4-desmethyl sterol which comprises extracting sterols from a plant of the invention.

In a further aspect, the invention provides a product comprising an oil produced by a process according to the invention.

A yet further aspect of the invention is the use of a gene expressing a C4SMO to increase the level of sterols in a plant.

Detailed description of the invention

Isoprenoids are a large family of compounds which are present in higher plants and which have diverse roles. They include sterols, the plant hormones gibberellins and abscisic acid, components of photosynthetic pigments, phytoalexins and a variety of other specialised terpenoids.

Sterols, especially 4-desmethylsterols are of interest because they contribute to the nutritional quality, flavour and colour of fruits and vegetable oils. Of particular interest are compounds of nutritional benefit such as fat-soluble sterols. These may be efficacious in reducing coronary heart disease, for example, some phytosterols have been shown to lower serum cholesterol levels when increased in the diet and vitamin E reduces atherosclerotic plaques via decreased oxidation of LDL.

Expression of such compounds in plant seeds, in particular in oilseeds, is commercially advantageous as generally the harvesting of such ingredients from seeds is very convenient and, in some instances, it may be possible to extract the oil in combination with the sterols from the seed, leading to an oil containing elevated levels of sterol without, or with the reduced need for, separate addition of sterols in order to obtain a nutritional benefit.

Preferred sterols are 4-desmethylsterols and their mixtures, most preferred are betasitosterol, sitostanol, stigmasterol, brassicasterol, campestanol, isofucosterol, campesterol, episterol and even more preferred sterols are sitosterol, stigmasterol, brassicasterol, avenosterol and campesterol. Also preferably, at least part of the sterols, for example at least 70 wt% based on the total weight of the sterols in the seed are esters of sterols with C10-24 fatty acids.

As discussed above, several approaches have been suggested to alter levels of isoprenoids in plants.

It has now been found that the level of 4-desmethyl sterols in plants can be increased by increasing the enzymatic demethylation of 4-monomethyl and 4,4-dimethyl sterols. It has not previously been recognised that the demethylation of 4-monomethyl and 4,4-dimethyl sterols can be a limiting step in the synthesis of the latter compounds and that other methods of increasing the production of 4-desmethyl sterols can therefore result in the build up of

intermediate 4-monomethyl and 4,4-dimethyl sterols rather than the desired 4-desmethyl sterols.

Increasing the enzymatic demethylation of 4-monomethyl and 4,4-dimethyl sterols in the invention can be carried out by treatment and/or modification of the plant in a number of ways that result in increased demethylation compared to plants not so treated and/or modified. The method may involve, for example, an increase in the expression and/or activity of the homologous enzyme responsible for the demethylation and/or the expression of an heterologous gene encoding a demethylase enzyme.

Preferably, the enzymatic demethylation is increased by increasing the activity of C-4 sterol methyl oxidase (C4SMO) in the plant. It is particularly preferred that the activity of C4SMO in the plant is increased by increased expression of a gene coding for C4SMO.

The term C4SMO and related terms, as used herein, refers to any polypeptide, including enzymes, fragments or variants of the enzymes (eg, allelic variants or mutants obtainable by insertion, deletion or substitution of one or more (eg, 1 to 5) amino acid residues), or precursors for the enzymes, in which the polypeptide has C-4 sterol methyl oxidase activity. The determination of whether a polypeptide exhibits C-4 sterol methyl oxidase activity can be readily carried out by the skilled person.

If the method of the invention involves increased expression of a C4SMO gene which is naturally present in the plant (ie, a homologous gene), the parameters and other factors controlling expression are then altered such that

increased expression of C4SMO, preferably in the seed region of the plant, will take place. For example, a suitable method involves the upregulation of facilitating molecules, such as transcription factors. Alternatively or additionally, a specific promoter can be inserted into the plant genome to ensure that the C4SMO gene is upregulated. A further example of a suitable method involves increasing the copy number of the "homologous" C4SMO gene to increase the expression thereof.

Alternatively, the C4SMO gene can be a heterologous gene, for example derived from other plant, animal or microbial sources. For example, the C4SMO gene may be derived from *Arabidopsis*, tobacco or yeast. The gene coding for C4SMO is preferably derived from *Arabidopsis* such as, for example, *Arabidopsis thaliana*. DNA segments encoding C4SMO for use according to the present invention may suitably be obtained from animals, microbial sources or plants. Alternatively, equivalent genes could be isolated from gene libraries, for example by hybridisation techniques with DNA probes. An example of C4SMO and the gene coding for C4SMO is given in Figure 2.

The gene sequences coding for C4SMO will be operably linked (that is, positioned to ensure the functioning of) to one or more suitable promoters which allow the DNA to be transcribed. Suitable promoters, which may be homologous or heterologous to the gene, useful for expression in plants are well known in art, as described, for example, in Weising et al, (1988), Ann. Rev. Genetics, 22, 421-477). Promoters for use according to the invention may be inducible, constitutive or tissue-specific or have various combinations of such characteristics. Useful promoters include, but are

not limited to constitutive promoters such as carnation etched ring virus (CERV), cauliflower mosaic virus (CaMV) 35S promoter, or more particularly the double enhanced cauliflower mosaic virus promoter, comprising two CaMV 35S promoters in tandem (referred to as a "Double 35S" promoter).

It may be desirable to use a tissue-specific or developmentally regulated promoter instead of a constitutive promoter in certain circumstances. A tissue-specific promoter allows for overexpression in certain tissues without affecting expression in other tissues. By way of illustration, a preferred promoter used in overexpression of enzymes in seed tissue is an ACP promoter as described in WO 92/18634.

The promoter and termination regulatory regions will be functional in the host plant cell and may be heterologous (that is, not naturally occurring) or homologous (derived from the plant host species) to the plant cell and the gene. Suitable promoters which may be used are described above.

The termination regulatory region may be derived from the 3' region of the gene from which the promoter was obtained or from another gene. Suitable termination regions which may be used are well known in the art and include *Agrobacterium tumefaciens* nopaline synthase terminator (Tnos), *Agrobacterium tumefaciens* mannopine synthase terminator (Tmas) and the CaMV 35S terminator (T35S). Particularly preferred termination regions for use according to the invention include the pea ribulose bisphosphate carboxylase small subunit termination region (TrbcS) or the *Tnos* termination region.

Such gene constructs may suitably be screened for activity by transformation into a host plant via *Agrobacterium* and screening for increased 4-desmethyl sterol levels.

Suitably, the nucleotide sequences for the genes may be extracted from the Genbank nucleotide database and searched for restriction enzymes that do not cut. These restriction sites may be added to the genes by conventional methods such as incorporating these sites in PCR primers or by sub-cloning.

Preferably the DNA construct which may be used in the invention is comprised within a vector, most suitably an expression vector adapted for expression in an appropriate host (plant) cell. It will be appreciated that any vector which is capable of producing a plant comprising the introduced DNA sequence will be sufficient.

Suitable vectors are well known to those skilled in the art and are described in general technical references such as Pouwels et al, Cloning Vectors. A laboratory manual, Elsevier, Amsterdam (1986). Particularly suitable vectors include the Ti plasmid vectors.

Transformation techniques for introducing the DNA constructs according to the invention into host cells are well known in the art and include such methods as Agrobacterium-mediated transfer, micro-injection, using polyethylene glycol, electroporation, or high velocity ballistic penetration.

After transformation of the plant cells or plant, those plant cells or plants into which the desired DNA has been

incorporated may be selected by such methods as antibiotic resistance, herbicide resistance, tolerance to amino-acid analogues or using phenotypic markers.

Various assays may be used to determine whether the plant cell shows an increase in gene expression, for example, Northern blotting or quantitative reverse transcriptase PCR (RT-PCR). Whole transgenic plants may be regenerated from the transformed cell by conventional methods. Such transgenic plants having improved isoprenoid levels may be propagated and self-pollinated to produce homozygous lines. Such plants produce seeds containing the genes for the introduced trait and can be grown to produce plants that will produce the selected phenotype.

Preferably, the levels of 4-monomethyl sterols and 4,4-dimethyl sterols in the plant are reduced. Thus, the proportion of 4-desmethyl sterols relative to 4-monomethyl and 4,4-dimethyl sterols is preferably increased in the method of the invention, compared to plants not having increased enzymatic demethylation.

Preferably the level of 4-desmethyl sterols, for example in the seeds of the plants, is at least 5wt% more than the level in corresponding plants without increased enzymatic demethylation based on the total weight of sterols, more preferably more than 7wt% more. The level of 4-monomethyl sterols, for example in the seeds of the plants, is preferably less than 75 wt% of the level in corresponding plants without increased enzymatic demethylation based on the total weight of sterols, more preferably less than 50 wt%. The total level of 4-desmethyl sterols in the seeds of the plants is preferably at least 80 wt%, more

preferably at least 85 wt% based on the total weight of sterols.

The levels of sterol intermediates in the plants, for example in the seeds or leaves of the plants can be altered compared to wild type plants. The weight ratio of cycloartenol (CA) to 24-methylenecycloartanol (24MCA) in plants of the invention, for example in the leaves, is preferably at least 3:1, more preferably at least 4:1, even more preferably at least 5:1, most preferably in the range 6:1 to 20:1.

The invention also contemplates seeds obtained from plants having increased enzymatic demethylation of 4-monomethyl and 4,4-dimethyl sterols. Especially preferred oilseeds are tobacco seeds, canola seeds, rapeseed, sunflower seeds and soybean seeds. Any suitable method can be used to extract oil from these seeds.

The plant of the invention is preferably tobacco, canola, sunflower, rape or soy.

The method of the invention is preferably used for the enhancement of 4-desmethyl sterol levels in plants in which the plant has been modified to increase the production of 4-monomethyl and/or 4,4-dimethyl sterols compared to the wild type plant. For example, the plant may have increased HMGR activity compared to the wild type plant. Alternatively or additionally to increased HMGR activity, the plant may have increased SMT1 activity compared to the wild type plant.

For instance, the plant may have been modified so as to incorporate a non-feedback inhibited HMGR gene in combination with sterol methyltransferase1. The use of such a combination of genes, together with the method of the invention, is especially advantageous to enhance the levels of 4-desmethylsterols, since the proportion in the end product of intermediate compounds relative to the desired 4-desmethylsterols can be reduced.

The non-feedback inhibited HMG reductase may be an enzyme which is expressed by a truncated non-plant HMGR gene, said truncation preferably leading to an enzyme lacking the membrane binding domain, but whereby the HMGR functionality of the gene is preferably maintained. Examples of such genes are the truncated hamster or yeast HMGR genes.

A second example of a non-feedback inhibited HMG reductase is an enzyme expressed by HMGR genes from high isoprenoid producing plants such as *Hevea brasiliensis*. Especially preferred are truncated versions of HMGR produced by genes from high isoprenoid producing plants such as *Hevea brasiliensis*, most preferred truncated versions are used whereby said HMGR lacks the membrane binding domain.

The intact HMGR enzyme comprises three regions: a catalytic region, containing the active site of the enzyme, a membrane binding region, anchoring the enzyme to the endoplasmic reticulum and a linker region joining the catalytic and membrane binding regions of the enzyme. The membrane-binding domain occupies the N-terminal region of the enzyme, whereas the catalytic region occupies the C-terminal region. It is believed that feedback inhibition in most plants generally requires the presence of the

membrane-binding region of the enzyme. Therefore it is preferred to use an HMGR gene expressing an enzyme with an inactivated or without a membrane binding domain, whereby said gene is preferably used to increase the level of 4-desmethylsterols in plant tissue such as the seeds of plants.

Suitable truncated HMGR genes are described in WO 01/31027, the contents of which are incorporated by reference herein.

Preferably the HMGR gene is isolated from *Hevea brasiliensis*. Especially preferably truncated versions of such plant genes may be used. A specific promoter can be inserted into the plant genome to ensure that the HMGR gene is upregulated, preferably within the seed tissue of the plant.

The plants may have been modified so as to have increased SMT1 activity. Plants having increased SMT1 activity are disclosed in WO 01/79513, the contents of which are incorporated by reference herein.

Suitably the SMT1 gene can be naturally present in the plant. The circumstances are then altered such that increased expression of SMT1, preferably in the seed region of the plant will take place. Possible ways to do this may be to upregulate facilitating molecules e.g. such as transcription factors. Alternatively, a specific promoter can be inserted into the plant genome to ensure that the SMT1 gene is upregulated. Alternatively, the copy number of the "homologous" SMT1 gene may be increased to increase the expression thereof.

Alternatively, the SMT1 gene can be a heterologous gene, for example derived from other plant or microbial sources. For example, the SMT1 gene may be derived from Arabidopsis, tobacco or yeast.

The plants may be modified to have increased HMGR activity and increased SMT1 activity. For example, WO 02/42477, the contents of which are incorporated herein by reference, discloses plants expressing genes encoding specific HMG-reductase enzymes (HMGR) in combination with those encoding sterol methyltransferase1

Cholesterol is a less desired component of food products because consumers have a desire to reduce their cholesterol consumption. It is believed that reduced serum cholesterol levels lead to a reduced risk of cardiovascular disease. Therefore, the invention preferably results in a reduction of the cholesterol level in plant tissue, especially the seeds of plants, in particular oilseeds containing more than 10 wt% based on dry weight of triacylglycerols.

Plants of the invention have increased levels of 4-desmethyl sterols compared to the wild type plant. Preferably, the plants have an increased proportion of 4-desmethyl sterols relative to 4-monomethyl and 4,4-dimethyl sterols compared to the wild type plant. More preferably, the increased levels and proportions are in the leaves and/or the seeds, particularly the seeds.

The process for producing an oil according to the invention comprises extracting sterols from a plant according to the invention. Methods for extracting oils from plants are well known to those skilled in the art. Preferably, the

oil is extracted from the seeds of the plant. The seeds are typically obtained by cultivating the plant for one or more generations and harvesting the seeds.

The oil may comprise, in addition to sterols, other compounds that are typically present in the plant, such as triglycerides. Alternatively, the oil may be subjected to one or more purification steps in order to increase the amount of sterols, and particularly 4-desmethyl sterols, in the oil.

Plant material obtainable from a plant of the invention may take the form of any part of the plant, including roots, leaves, stems and seeds. Preferably, the plant material is a leaf or seed, more preferably a seed. The plant material may be the leaves or seeds as obtained directly from the plant or may have undergone one or more further processing steps such as, for example one or more of washing, drying, milling, grinding and heat treating.

The product comprising an oil of the invention may be suitable for use in one or more of a number of different applications. For example, the product may be a food product, an oil for use in food preparation, a lubricating oil, a fuel oil or a feedstock for use in the production of hydrocarbons. The product may comprise additives suitable for the intended application of the product eg, food preservatives and/or stabilisers when the product is a food product. The product may comprise the oil as a single phase or the oil can be dispersed, suspended or emulsified in another liquid eg, as a water-in-oil or oil-in-water emulsion.

The invention will now further be illustrated in the following non-limiting examples. In the examples and throughout this specification, all references to percentages are percentages by weight based on total weight unless indicated otherwise.

The examples refer to the accompanying drawings in which:

Figure 1 shows the sterol biosynthesis pathway post cycloartenol highlighting C4-demethylation steps. Solid lines indicate a single, and the dashed lines more than one, catalytic conversion.

Figure 2 shows the putative open reading frame of *AtC4SMO* and the corresponding translated protein. The histidine rich motives are underlined.

Figure 3 shows real time PCR analysis of the *AtC4SMO* transcription levels in leaf tissue of representatively selected NH65 lines.

Figure 4 shows the CA : 24MCA, CA : 24Mloph and CA : 24Eloph ratios in mature seed of selected NH65 lines. The error bars correspond to the standard deviation.

Figure 5 shows the real time PCR analysis of the *AtC4SMO* transcription levels in leaf tissue of NH65 and MH7xNH65 lines.

Figure 6A shows the CA/24MCA, CA/24Mloph and CA/24Eloph ratios in mature leaf tissue of selected MH7xNH65 lines. Figure 6B shows the CA/24MCA, CA/24Mloph and CA/24Eloph

ratios in mature seed tissue of selected MH7xNH65 lines. The error bars correspond to the standard deviation

Examples

Experimental procedures

Strains and plasmids

E. coli strain DH5α (Gibco BRL) was used as the host strain in all cloning procedures. *E. coli* were cultivated in LB medium (10 g/L tryptone, 5g/L yeast extract, 5 g/L NaCl) supplemented with the appropriate selection pressure (ampicillin 100 µg/mL or kanamycin 50 µg/mL) on a rotary shaker (210 rpm) at 37°C.

PCR cloning vector pGEM-T easy was obtained from Promega. Binary vector pSJ35 was created by filling in the *Bam*HI restriction site of the pGPTV-HYG with the Klenow enzyme (Becker et al., Plant Mol. Biol. 20, 1195-1197, 1992). Plasmid pNH2, harbouring the carnation etched ring virus (CERV) promoter and the nopaline synthase (NOS) terminator, has previously been described in WO 01/31027 (see Example 4).

Enzymes and chemicals

Restriction endonucleases, T4 DNA ligase, molecular markers (X, XIV and XVII) and Taq DNA polymerase were purchased from Roche. Pfu DNA polymerase was obtained from Stratagene. The enzymes were used according to the suppliers' recommendations. Biochemicals were purchased

from Sigma Chemical Co. All chemicals and reagents used were of analytical grade and available from either Fisher Scientific UK or BDH.

Plant material

Tobacco SR1 (Petite Havana) was grown in either MS-medium or a compost/perlite mixture (2:1) (Murashige and Skoog, Physiol Plant 15, 473-497, 1962). The temperature in the growth rooms was kept at 22°C, and a day/night cycle of 16h / 8h was used. The light intensity was 40 µmol m^{-2} s^{-1}.

Oligonucleotide synthesis

All oligonucleotides were synthesised and they are compiled in Table 1.

Table 1. Oligonucleotide primers (given 5' to 3' direction)

Primer	Sequence
CERV1S	*gtc tgt cta aag taa agt aga tgc g*
C4SO1	*tac ctt gtt acg cat ttc a*
C4SO2	*tag ggc ctt aag ttt tct gt*
clC4SO1[a, b]	*c cca agc ttc* aaa ***ATG*** *ATG CAG TAC CTT GTT ACG*
clC4SO2[c]	*gg aat TCA GGT TTC TTT TAG GGC CTT AAG TTT TCT G*
clC4SOp1[d]	*cc cac* ***ATG*** *tTG CAG TAC CTT GTT ACG*
clC4SOp2[e]	*c tca tag agc TCA GGT TTC TTT TAG GGC CTT AAG*
C4SO3	*act gga tgg atg gtg tca a*
C4SO4	*agt ggg att tat gta ttg ttg ttg*
NosAs	*ccg gca aca gga ttc aat ctt*
RoRidT17	*aag gat ccg tcg aca tcg ata ata cga ctc act ata ggg att ttt ttt ttt ttt ttt ttt*
181	*gga aac agc tat gac cat gat tac*
M13/pUC Forward	*ttt ccc agt cac gac gtt gt*
M13/pUC Reverse	*gta aaa cga cgg cca gt*
TaqA1	*tgc tga gcg ttt ccg ttg*
TaqA2	*ccg gca gct tcc att cc*
TaqC4S1	*gtg cac agt gtg cat cat gag t*
TaqC4S2	*ttc agc ggg atg agc ata ttc*

a, Introducing a *Hind*III (underlined). b, introducing a yeast translation initiation context (italics). C, Introducing an *Eco*RI site (underlined). d, *Afl*III (underlined). e, *Sac*I, (underlined).

Cloning *C4SMO* from *Arabidopsis thaliana*

The primary sequence of the ERG25 protein from yeast was used as the query sequence in a BLAST search of all non-redundant proteins in the *Arabidopsis* database (located at Stanford). This approach rendered a putative *Arabidopsis thaliana C4SMO* with the accession number At2g29390.

Messenger RNA from 12 day old *Arabidopsis thaliana* (eco-type Columbia) seedlings was isolated using the Pharmacia QuickPrep micro mRNA purification kit according to the supplier's recommendations. The mRNA was isolated based on affinity to oligo(dT) coated cellulose. First strand DNA was synthesised by mixing Poly-A RNA (1 µg) with primer RoRidT17 (10 pmol) in 11.34 µL DEPC treated water. The mixture was incubated at 70°C for 10 min and thereafter placed in wet ice for 2 min. First strand buffer (1X), DTT (0.1µmol), RNAsin (22 U), dNTP (20 nmol) and Superscript (200 U) was added to give a final volume of 20 µl. The mix was incubated at 37°C for 60 min to give a pool of *Arabidopsis* cDNA.

The putative *AtC4SMO* gene was amplified from a pool of *Arabidopsis* cDNA or genomic DNA using PCR and gene specific primers C4SO1 and C4SO2 (Table 1). The following amplification program was used: 1 cycle 94°C (2 min), 5 cycles 94°C (30 s), 40°C (30 s), 72°C (2 min), 30 cycles 94°C (30 s), 40°C (30 s), 72°C (90 s) and 1 cycle 72°C (7 min), 4°C (Hold). Proof reading enzyme Pfu Turbo DNA polymerase was used to minimise the number of errors introduced. The amplified fragments (from cDNA or genomic

DNA) were cloned into PCR product cloning vector pGEM-T Easy according to the supplier's instructions (Promega). Clones containing either putative *AtC4SMO* CDNA or genomic *AtC4SMO* fragments were selected and sequenced using primers M13/pUC universal forward and reverse primers, and C4SO1 and C4SO2.

Plant expression vectors

AtC4SMO was amplified by PCR using primer pair clC4SOp1 / clC4SOp2 to introduce restriction enzyme sites *Afl*III and *Sac*I in the 5' and 3' end of the gene, respectively. The amplification reaction was performed under standard conditions using the proof reading enzyme Pfu turbo DNA polymerase to minimise errors. The resulting amplification product was purified, digested by *Afl*III and *Sac*I and inserted into pNH2, downstream of the constitutive carnation etched ring virus (CERV) promoter and upstream of the nopaline synthase (NOS) terminator, yielding pNH64. The expression cassette from pNH64 was excised by digestion (*Hind*III and *Eco*RI) and inserted into the corresponding sites in pSJ35 giving pNH65. Vector pNH64 was sequenced using primers CERV1S, clC4SOp1, clC4Sp2, and NosAs, and pNH65 was sequenced using primers CERV1S and NosAs to confirm their authenticity.

Sequencing

All plasmid DNA used for sequencing was purified using the Qiagen mini spin kit. Sequencing was performed with fluorescently labelled nucleotides using the ABI 377 sequencer.

Plant transformation

Binary vectors were transformed via electroporation into *A. tumefaciens* LBA4404 (Shen and Forde, Nucl. Acids Res. 17, 8385, 1989). *Nicotiana tabacum* cv. SR1 was transformed via the leaf disc method as described by An et al, Plant Physiol. 88: 547-552 (1988). Transgene containing plants were selected by PCR using primer pair CERV1S/NosAs. These plants were transferred into soil.

Transcript analysis

Total RNA was isolated from young (7 to 8 leaf stage) NH65 and SR1 plants using the RNaqueous kit according to the supplier's instructions (Ambion, Austin, USA). The total RNA was treated with DNase to remove any contamination of genomic DNA and subsequently converted into cDNA using the 3'-RACE System from Gibco-BRL (Life Technologies Ltd., UK). Taqman primer pairs directed against *AtC4SMO* (TaqC4S1 and TaqC4S2) and tobacco *tac9* actin (TaqA1 and TaqA2) genes were designed using the Primer Express software. These primer pairs were used together with Sybr Green (Applied Biosystems, USA) in Taqman PCR reactions to detect transcript levels of *AtC4SMO* and *tac9* in transgenic and control samples. The *AtC4SMO* transcript level in the transgenic tobacco was calculated in relation to the transcript levels in SR1 tobacco according to the manual supplied by Applied Biosystems.

Sterol analysis

Mature leaf and seed tissues were freeze-dried and extracted in chloroform / methanol 2:1(v/v) at 80°C. After filtration and removal of solvent, the lipid residue was dissolved in toluene followed by sodium methoxide to a

concentration of 0.33M. The mixture was heated for 30 min at 80°C followed by a further 10 min at 80°C in the presence of 5.6% (w/v) boron trifluoride. Following diethyl ether extraction and washing with water, the ether was evaporated and the free sterols were silylated by addition of trimethylchlorosilane / N, O-bis (trimethylsilyl) acetamide (5:95) and heating for 10 minutes at 50°C. GC analysis was carried out using a Perkin Elmer 8420 GC equipped with a BPX5 column. The temperature programme was 80-230°C at 45°C / minute, followed by 230-280°C at 4°C / minute and 355°C for 6 minutes. Peak areas were calculated automatically using Turbochrom software. Identity of sterols was confirmed by GC-MS, using a Hewlett Packard 5890 GC coupled to a Quadrapole 5972A MSD.

Example 1

Cloning *C4SMO* from *Arabidopsis*

The primary sequence of C4SMO from yeast (ERG25) was used as a probe to search the *Arabidopsis thaliana* proteome to identify homologous proteins. A putative C4SMO (At2g29390) was found which exhibited 37% identity with ERG25. PCR primers were designed to amplify the corresponding *AtC4SMO* gene from pools of *Arabidopsis* cDNAs or genomic DNA. The cDNA clone comprised a 762 bp open reading frame (ORF), which encoded a 253 amino acid protein (Figure 2). The predicted molecular weight of AtC4SMO was 29.5-kDa. This is similar not only to the ERG25 protein (36.5-kDa) but also to the 29-kDa C4SMO purified from rat liver microsomes (Maitra et al.,Biochem. Biophys. Res. Commun. 108: 517-525, 1982). The primary sequence of AtC4SMO contains four histidine-rich motifs (H^{139}RILH, H^{152}SVHH, H^{210}CGYH, H^{232}DYHH), indicative of iron non-heme enzymes (Shanklin et al,

Biochem. 33: 12787-12794, 1994). It has previously been shown that ERG25 belongs to the same class of enzymes (Bard et al., Proc. Natl. Acad. Sci. USA 93: 186-190, 1996), which further supports the conclusion that AtC4SMO is a plant homologue of ERG25.

The genomic sequence was aligned with the putative *AtC4SMO* ORF revealing a splicing-pattern, which comprised 6 exons and 5 introns. The primary sequence of AtC4SMO was analysed for the presence of a signal peptide and transmembrane spanning domains using the SeqWeb software package (Genetic Computer Group Inc). AtC4SMO was shown to have a possible amino-terminal signal peptide, which may direct translocation of the polypeptide to the endoplasmic reticulum (ER). However, the carboxy-terminus does not contain the characteristic KDEL motif required for retention in the ER lumen. An unequivocal cleavage site could not be identified but three likely transmembrane spanning domains were identified using hydrophobicity plots (amino acids 64-86, 99-121, 154-176). Together, these predictions suggest that AtC4SMO is an integral membrane protein located to the ER membrane. This is consistent with the localisation of other enzymes involved in sterol biosynthesis, such as HMGR (Bach et al., Crit. Rev. Biochem. Mol. Biol. 34: 107-122, 1999).

Overexpression of AtC4SMO in wild type tobacco

AtC4SMO was placed under control of the constitutive carnation etched ring virus (CERV) promoter upstream of the nopaline synthase (NOS) terminator, giving binary vector pNH65. Wild type tobacco was transformed with this vector using the leaf disc method and transformants selected on hygromycin (25mg / L). Thirty transgenic plants were

selected by PCR. The transcription of *AtC4SMO* in selected transgenic lines was analysed using real time PCR. As shown in Figure 3 all NH65 lines analysed exhibited elevated *AtC4SMO* expression. The highest expressing line, NH65:16, displayed transcript levels that were 23-fold higher than the average of the wild type controls.

The sterol content in leaf and seed tissue of the NH65 lines was analysed. In order to assess the effect of AtC4SMO expression, the ratio of cyloartenol (CA) to 24-methylene cycloartanol (24MCA), 24-methylene lophenol (24Mloph) or 24-ethlidene lophenol (24Eloph) was calculated. Sterol accumulation in leaf is highly dependent on the age of the tissue (Chappell et al, Plant Physiol. 109, 1337-1343, 1995; Schaller et al, Plant Physiol. 118: 461-469, 1995), which is clearly reflected in the varying amount of total sterol found in maturing tobacco leaves but calculation of ratios overcomes this issue. Furthermore, since CA is the first sterol-intermediate, the amount of CA reflects the flux of carbon partitioned into sterol biosynthesis. The ratio of CA to 24MCA, 24Mloph or 24Eloph will hence increase if the relative levels of any of the C4SMO substrates have been reduced.

The levels of CA, 24MCA, 24Eloph and total sterol were measured in leaf tissue of five independent SR1 and three NH65 lines (Table 2).

Table 2. *Levels of cycloartenol (CA), 24-methyl cycloartanol (24MCA), 24-ethylidene lophenol (24Eloph) and total sterols in wild type and AtC4SMO expressing tobacco leaves*

Line	CA	24MCA	24Eloph	Total sterols	CA : 24MCA	CA : 24Eloph
SR1[a]	0.0110[b]	0.0042	0.0037	0.1850	2.619	2.973
(stdev)	(0.0064)	(0.0018)	(0.00017)	(0.034)	(0.511)	(1.709)
NH65:7	0.0113	0.0011	n.d.[c]	0.2570	10.273[d]	11.300
NH65:18	0.0094	0.0021	n.d.	0.2170	4.476	9.400
NH65:16	0.0098	n.d	n.d.	0.2610	9.800	9.800

a, The average and standard deviation is calculated based on five independent SR1 plants. b, % of dry weight. c, The detection limit is <0.001 % of dry weight. d, The ratios of CA : 24MCA and CA : 24Eloph are calculated using a value of 0.001% of dry weight.

The levels of 24Mloph were below the detection limit (<0.001% of dry weight) and were therefore excluded. The three NH65 lines were chosen on the basis of high *AtC4SMO* transcript levels (Figure 3). The absolute amount of 24MCA was reduced in all NH65 lines analysed (Table 2). The CA : 24MCA ratio of the NH65 lines spanned between 4.5 (NH65:18) and 10.3 (NH65:7), which is considerably higher than the wild type (SR1) value (2.6). This suggests that the putative AtC4SMO is able to convert 24MCA into downstream products. The absolute amount of 24Eloph was undetectable (<0.001% of dry weight) in the leaf tissue of all three NH65 lines whereas the average value of the wild type controls was 0.0037% of dry weight (Table 2). The detection limit, 0.001% of dry weight, was used to estimate the CA : 24Eloph ratio when 24Eloph was undetectable. The ratio of CA : 24Eloph was dramatically increased in the NH65 lines (up to 11.3) compared to the wild type control (2.9) (Table

2). This demonstrated that AtC4SMO can also catalyse the conversion of 24Eloph into downstream sterol products.

The sterol compositions of seed tissue of ten independent NH65 lines were also analysed. As shown in Table 3 the levels of 24MCA and 24Eloph were significantly reduced in all ten NH65 lines, whilst the level of 24Mloph was not significantly altered. Calculating the ratios of CA : C4SMO substrate also reflected these changes. The CA : 24MCA ratio was more than doubled in the top five NH65 lines and the CA : 24Eloph ratio was up to 4-fold higher in the top NH65 line compared to the wild type tobacco (Figure 4). However, the CA : 24Mloph ratio remained unchanged in the NH65 lines compared to the control.

Table 3 Levels of cycloartenol (CA), 24-methyl cycloartanol (24MCA), 24-methylene lophenol (24Mloph), 24-ethylidene lophenol (24Eloph) and total sterols in wild type and AtC4SMO expressing tobacco seed

Line	CA	24MCA	24Mloph	24Eloph	Total sterols
SR1[a]	0.0398[b]	0.0038	0.0072	0.0379	0.414
(stdev)	(0.00059)	(0.00026)	(0.0022)	(0.015)	(0.022)
NH65:48	0.0377	0.0018	0.0072	0.0143	0.443
NH65:18	0.051	0.0023	0.0093	0.0170	0.458
NH65:27	0.0510	0.0023	0.0093	0.0170	0.449
NH65:33	0.0483	0.0023	0.0086	0.0176	0.475
NH65:41	0.0300	0.0023	0.0073	0.0181	0.389
NH65:36	0.0374	0.0024	0.0084	0.0183	0.452
NH65:7	0.0419	0.0020	0.0088	0.0251	0.470
NH65:10	0.0357	0.0022	0.0079	0.0251	0.405
NH65:42	0.0310	0.0023	0.0081	0.0265	0.426
NH65:16	0.0385	0.0025	0.0086	0.0284	0.432

a, The average and standard deviation is calculated based on five independent SR1 plants. b, % of dry weight.

In seed AtC4SMO preferentially catalyses conversion of 24MCA and 24Eloph with 24Mloph levels being unchanged. In leaf AtC4SMO also catalyses conversion of 24MCA and 24Eloph into downstream sterols, but since 24Mloph is not present in detectable levels in this tissue it was not possible to determine if conversion of 24Mloph occurs.

Altered distribution of 4,4-dimethyl-, 4-mono- and 4-desmethyl sterols in tobacco seed over-expressing C4SMO

The relative distributions of the most abundant di-, mono- and des-methyl sterols in seed tissue were calculated for wild type (SR1 and SJ35) and NH65 lines. The 4,4-dimethyl sterols include cycloartenol and 24-methylene cycloartanol,

the 4-monomethyl sterols include 24-methylene lophenol and 24-ethylidene lophenol, whilst the 4-desmethyl sterols include Δ7-avenasterol, isofucosterol, sitosterol, stigmasterol, campesterol and cholesterol. As shown in Table 4 the relative level of 4,4-dimethyl sterols is not significantly different in the seed of the NH65 lines as compared to the wild type controls. However, the relative levels of 4-monomethyl sterols are reduced in all bar one of the NH65 lines compared to the control levels. Furthermore, 23 out of 25 NH65 lines have elevated relative levels of 4-desmethyl sterols. In addition, a strong correlation between *AtC4SMO* transcription and sterol profile was observed as line NH65:16 exhibited the lowest relative level of 4-monomethyl sterols and the highest relative level of 4-desmethyl sterols and the highest *AtC4SMO* transcription level (Figure 3, Table 4).

Table 4. Relative levels of the 4,4-di-, 4-mono- and 4-desmethyl sterols in tobacco over-expressing AtC4SMO

Sample	Dimethyl sterol[b] (%)[e]	Monomethyl[c] sterol (%)	Desmethyl[d] sterol (%)
SR1[a]	10.5 ± 0.4	11.2 ± 0.6	78.3 ± 0.9
SJ35[a]	9.8 ± 0.5	10.9 ± 0.2	79.3 ± 0.5
NH65:8	12.8	8.3	78.9
NH65:18	10.0	10.0	79.9
NH65:22	9.4	10.6	80.0
NH65:4	9.8	9.9	80.3
NH65:22	9.4	10.1	80.5
NH65:40	9.9	9.3	80.8
NH65:46	9.7	9.2	81.1
NH65:47	9.7	8.7	81.7
NH65:28	9.7	8.5	81.8
NH65:30	9.4	8.7	81.9
NH65:10	9.5	8.6	81.9
NH65:20	9.0	9.0	82.1
NH65:23	9.5	8.3	82.1
NH65:19	8.8	9.0	82.2
NH65:27	11.9	5.8	82.3
NH65:1	10.3	7.4	82.3
NH65:33	10.9	5.6	83.5
NH65:7	9.9	6.5	83.5
NH65:42	8.0	8.3	83.7
NH65:50	8.1	8.1	83.8
NH65:37	9.2	6.6	84.2
NH65:41	8.5	6.7	84.9
NH65:36	9.0	6.1	84.9
NH65:48	9.1	5.0	85.9
NH65:16	9.8	4.3	85.9

a, The average and standard deviation of is calculated based on five independent SR1 and SJ35 lines. b, Dimethyl sterols include cycloartenol and 24-methylene cycloartanol. c, Monomethyl sterols include 24-methylene lophenol and 24 ethylidene lophenol. d, Desmethyl sterols include Δ7-avenasterol, isofucosterol, sitosterol, stigmasterol, campesterol and cholesterol. e, Calculated as % of total sterols.

Example 2: Co-expression of C4SMO, tHMGR and SMT1 in transgenic tobacco

Overexpressing AtC4SMO in a high sterol background

A tobacco plant co-expressing of a truncated form of *Hevea brasiliensis hmg1* and *Nicotiana tabacum* SMT1 was obtained as described in WO 02/42477.

A truncated form of *Hevea* HMGR, lacking the N-terminal membrane-binding domain, was cloned using the *Hevea brasiliensis hmg1* as template. The *Hevea brasiliensis* (H.B.K.) Müll. Arg. *thmg1* was cloned using the primers based on the published sequence [Chye et al (1991) Plant Mol Biol 19: 473-84]. The forward primer 5′-CCTACCTCGGAAGCCATGGTTGCAC-3′ incorporates a new start codon (bold) and a *Nco I* restriction site (underlined) for cloning applications. The reverse primer 5′-CATTTTACATTGCTAGCACCAGATTC-3′ contains a *Nhe I* restriction site (underlined) for downstream sub-cloning purposes. The

plasmid pNH8 was used as the template DNA in the PCR (30 cycles) using *Pfu* polymerase under standard conditions and produced a fragment of the expected size ~1.3 kb. The resulting *thmg1* gene codes for amino acids 153-575 of the full-length (575) *hmg1* sequence (Fig. 11b of PCT/EP/00/09374). The *thmg1* PCR product was cloned into the pGEM-T vector (Promega) according to the manufacturers' instructions and sequenced to confirm fidelity. The *H. brasiliensis tHMG1* was inserted into pNH4 (see PCT/EP/00/009374) between the *Nco I* and *Nhe I* sites of the polylinker, which lie between the CaMV 35S double promoter and nos terminator, giving pMH3 (see PCT/EP/00/09374). This chimaeric gene was isolated by digestion with *Xma CI* and *Sal I*, purified and cloned into the corresponding polylinker sites in pNH9, after removal of the chimaeric full length *hmg1* gene which previously occupied these sites, and subsequent purification of the binary vector. The binary vector pNH9 was derived by firstly inserting the Cerv promoter and nos terminator cassette from pNH2 into *Eco*RI and *Xma*I digested pSJ34, and subsequently placing the *Nicotiana tabacum* sterol methyl tramsferase type 1 (*Ntsmt1-1*) gene under transcriptional control of the Cerv promoter. The binary vector pNH9 also contains the *smt1* gene cloned from *Nicotiana tabacum*, which is under transcriptional control of the CERV viral promoter. The binary construct was named pMH7.

Electrocompetent *Agrobacterium tumefaciens* cells (strain LBA4404) were defrosted on ice and 5ng of vector plasmid added. Cells plus plasmid were then placed into a pre-chilled electroporation cuvette and electroporated in a Bio Rad Gene Pulser at a capacitance of 25μF and at 600 ohms.

Immediately after electroporation 950µl of 2X TY broth was added, the cells mixed gently and placed in a sterile vial. The cells were shaken at 28°C for 2 hours and 25µl aliquots plated on solid Lennox media containing rifampicin 50µg/ml and kanamycin 50µg/ml and incubated at 28°C for 3 days. Single colonies were used to inoculate 10µl of water (for PCR confirmation) and 500µl of Lennox media containing rifampicin 50µg/ml and kanamycin 50µg/ml.

PCR positive cultures were used to inoculate a 10 ml of Lennox media broth containing rifampicin 50µg/ml and kanamycin 50µg/ml. The overnight culture was spun down at 3000g and resuspended in an equal volume of MS media (3% sucrose). Leaf segments were cut from young tobacco leaves from plants grown in tissue culture. Segments were placed directly into the agrobacterium solution and left for 10 minutes. The segments were then removed and placed upper surface down on feeder plates (10 per plate) and left for 2 days in low light at 22°C. The leaf segments were placed, upper surface up, on tobacco shooting media with hormones containing cefotaxime 500µg/ml and kanamycin 50µg/ml and placed in a growth room at 24°C with a 16hrs light / 8 hrs dark regime. Three weeks later, the callusing segments were transferred to Magenta tubs containing tobacco shooting media. Once formed, shoots were excised and placed on tobacco shooting media containing cefotaxime 500µg/ml and kanamycin 50µg/ml without hormones, to root. Rooted plants were then potted up into a 50% perlite / 50% compost mixture and placed in a propagator. After 1 week the plants were removed from the propagator and subsequently potted up into 5 inch pots. Once flowering had begun paper bags were

placed over the flowers to prevent cross pollination. When flowering had finished and pods formed the bags were removed and mature pods harvested. Mature leaves and seed from dry pods were harvested and stored for subsequent analysis.

The resulting transgenic tobacco line MH7:53, co-expresses truncated *H. brasiliensis* HMGR and *N. tabacum* SMT1. It consequently over-accumulates intermediates that serve as substrates for sterol methyl oxidases (C4SMOs), i.e. 2-methylene cycloartanol (24MCA), 24-methylene lophenol (24Mloph) and 24-ethylidene lophenol (24Eloph) (Table 5).

MH7:53 leaf discs were re-transformed with NH65, expressing At C4SMO, as described above and transformants were selected by resistance to hygromycin (25mg/L). Twenty transgenic MH7xNH65 plants were selected by PCR. The levels of *AtC4SMO* transcription in selected MH7xNH65 lines were analysed using real time PCR. As shown in Figure 5 all MH7xNH65 lines analysed exhibited elevated *AtC4SMO* expression. The highest expressing line, MH7xNH65:10, expressed 17-fold more *AtC4SMO* transcripts than average of the wild type controls.

Table 5. Levels of cycloartenol (CA), 24-methyl cycloartanol (24MCA), 24 methylene lophenol (24Mloph), 24-ethylidene lophenol (24Eloph) and total sterols in wild type and tobacco leaves co-expressing tHMGR, SMT1 and AtC4SMO

Lines	CA	24MCA	24Mloph	24Eloph	Total sterols
SR1[a]	0.0110[b]	0.0042	n.d.[c]	0.0037	0.1850
(stdev)	(0.0064)	(0.0018)		(0.00017)	(0.034)
MH7:53 T1[a]	0.285	0.427	0.192	0.290	2.758
(stdev)	(0.007)	(0.024)	(0.029)	(0.033)	(0.008)
MH7xNH65:10	0.127	0.137	0.118	0.024	1.201
MH7xNH65:28	0.218	0.196	0.188	0.151	1.773

a, The average and standard deviation is calculated based on five independent SR1 lines and four independent MH7:53 lines. b, % of dry weight. c, the detection limit is <0.001% of dry weight.

Analysis of leaf tissue

The sterol content in mature leaf tissue of two high C4SMO expressing lines, MH7xNH65:10 and MH7xNH65:28, were analysed. The absolute levels of 24MCA in these lines were 0.137 to 0.196% of dry weight, respectively, which is much lower than the 24MCA level of the MH7:53 background (0.427% of dry weight) (Table 5). The CA : 24MCA ratios of MH7xNH65:10 and :28 were significantly higher than the MH7:53 background (Figure 6A). The levels of 24Mloph were significantly lower in line MH7xNH65:10 but not in line MH7xNH65:28 (Table 5). However, when the overall carbon fluxes were taken into account by calculating the CA :

24Mloph ratios both of these transgenic lines exhibited significantly higher ratios than the MH7:53 background (Figure 6A). The absolute amounts of 24Eloph were significantly lower in both line MH7xNH65:10 and MH7xNH65:28 compared to the MH7:53 background (Table 5). In addition, the CA : 24Eloph ratios of these transgenic plants were increased over the MH7:53 control (Figure 6A).

Analysis of seed tissue

The absolute levels of 24MCA and 24Mloph were not significantly altered in seed tissue of MH7xNH65:10 and MH7xNH65:28 lines compared to the parent MH7 line (Table 6).

Table 6. Levels of cycloartenol (CA), 24-methyl cycloartanol (24MCA), 24 methylene lophenol (24Mloph), 24-ethylidene lophenol (24Eloph) and total sterols in wild type and tobacco seed co-expressing tHMGR, SMT1 and AtC4SMO

Lines	CA	24MCA	24Mloph	24Eloph	Total sterols
SR1[a]	0.0398[b]	0.0038	0.0072	0.0379	0.414
(stdev)	(0.00059)	(0.00026)	(0.0022)	(0.015)	(0.022)
MH7xSJ35[a]	0.0739	0.140	0.0441	0.0743	0.874
(stdev)	(0.0.0042)	(0.015)	(0.0034)	(0.013)	(0.043)
MH7xNH65:10	0.0898	0.122	0.0374	0.0358	0.911
MH7xNH65:28	0.0963	0.122	0.0432	0.0521	0.846

a, The average and standard deviation is calculated based on five independent SR1 lines and four independent MH7xSJ35 lines. b, % of dry weight.

In contrast, the levels of 24Eloph were significantly lower in both these lines than the MH7 background. The ratios of CA to the C4SMO substrates broadly exhibited the same patterns in seed and leaf (Figures 6A and B). The main difference is that the CA : 24Mloph ratio was significantly higher than the parent MH7 in lines MH7xNH65:10 and MH7xNH65:18.

Taken together these results indicate that AtC4SMO catalyses the conversion of 24MCA and 24Eloph into downstream phytosterols. Furthermore, AtC4SMO also catalysed the conversion of 24Mloph in leaf but not seed tissue, which may be explained by differences in substrate

specificity or the fact that the overall carbon flux in the sterol biosynthesis pathway is higher in leaf than seed.

Altered distribution of 4,4-di-, 4-mono- and 4-desmethyl sterols in tobacco seed over-expressing HMGR, SMT1 and C4SMO

The relative distributions of the most abundant di-, mono- and des-methyl sterols were calculated for wild type (SR1), MH7 and two MH7xNH65 lines (:10 and :28). The 4,4-dimethyl sterols include cycloartenol and 24-methylene cycloartenol, the 4-monomethyl sterols include 24-methylene lophenol and 24-ethylidene cycloartenol, whilst the 4-desmethyl sterols include Δ7-avenasterol, isofucosterol, sitosterol, stigmasterol, campesterol and cholesterol. As shown in Table 7 the relative level of 4,4-dimethyl sterols is similar in the MH7xNH65 seed compared to the MH7 background. However, the relative levels of 4-monomethyl sterols are reduced in both MH7xNH65 lines compared to the background (MH7). Furthermore, both MH7xNH65 lines (:10 and :28) have elevated relative levels of 4-desmethyl sterols. In addition, a correlation between expression and sterol profile was observed as the line with highest level of AtC4SMO expression, MH7xNH65:10, also exhibited the lowest relative level of 4-monomethyl sterols and the highest relative level of 4-desmethyl sterols.

Table 7. Distribution of 4,4-di-, 4-mono- and 4-des-methyl sterols in MH7xNH65 tobacco seed

Sample	Dimethyl sterols (%)	Monomethyl sterols (%)	Desmethyl sterols (%)
SR1[a]	0.105±0.004	0.112±0.006	0.783±0.009
MH7xSJ35[a]	0.245±0.0165	0.136±0.0133	0.620±0.0102
MH7xNH65:28	0.258	0.113	0.630
MH7xNH65:10	0.232	0.080	0.688

a, The average and standard deviation is calculated based on five independent SR1 lines and five independent MH7xSJ35 lines.